Jpn. J. Cancer Res. (Gann), 79, 69–73 ; January, 1988

Membrane-associated Sialidase of Rat Liver and Its Decrease in Hepatomas

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Using the particulate fraction of tissue homogenate, plasma membrane-associated sialidase was assayed at pH 4.5 with bovine brain mixed gangliosides as the substrate. The activity was lower in rat hepatoma induced by 3′-methyl-4-dimethylaminoazobenzene (MeDAB) and transplantable AH-109A rat hepatoma than in normal rat liver. The enzyme was almost quantitatively solubilized from liver particulate fraction by using 0.5% (w/v) sodium deoxycholate plus 0.2% (w/v) Triton X-100. When chromatographed on DEAE-cellulose, the solubilized activity emerged as a single peak. The enzyme thus obtained was maximally active at pH 4.5, and readily hydrolyzed mixed gangliosides but was less active toward 4-methylumbelliferyl-α-N-acetylneuraminic acid, 3′-sialyllactose and fetuin. The corresponding enzyme from MeDAB-induced hepatoma was indistinguishable from the liver enzyme in terms of ease of solubilization, pH-activity relationship, chromatographic behavior and substrate preference. It therefore appears that the plasma membrane-associated sialidase of hepatomas differs from that of liver only in the tissue level of activity.

Key words: Sialidase — Gangliosides — Plasma membrane — Rat liver — Rat hepatoma

It has been suggested that cancer cell phenotypes such as invasiveness[1, 2] and metastatic potential[3] may be caused, at least in part, by excessive sialylation of the surface of these cells. In attempts to elucidate the mechanism of this aberrant sialylation, we have been studying sialidase (EC 3.2.1.18)[4–7] as well as sialyltransferase (EC 2.4.99.1)[8, 9] in rat liver and hepatomas. We have already established that rat liver possesses at least three types of sialidase, localized in the lysosomal matrix,[4] cytosol[5] and plasma membrane[6]; these sialidases also differ in substrate preference, though their physiological functions remain obscure. We have also shown that in rat hepatomas, the activity of intralysosomal sialidase is elevated but that of cytosolic sialidase is lowered as compared with normal rat liver.[7] No qualitative difference, however, has been detected for either enzyme before and after hepatocarcinogenesis.[7] The present paper deals with the solubilization, partial characterization and oncogenic alterations of the third type of sialidase that is associated with the plasma membrane of rat liver.

Abbreviation used: MeDAB, 3′-methyl-4-dimethylaminoazobenzene.

MATERIALS AND METHODS

Materials Bovine brain mixed gangliosides were purchased from Sigma (St. Louis, MO) and used as a substrate throughout the present studies. Sodium deoxycholate and Triton X-100 were the products of Calbiochem (La Jolla, CA). DEAE-cellulose (DE-52) and AG 1-X2 were obtained from Whatman (Kent) and Bio-Rad (Richmond, CA), respectively. The sources of other materials were described elsewhere.[4–7]

Liver and Hepatomas Fresh normal liver was obtained from male Wistar or Donryu rats (150–200 g) fed *ad libitum.* Primary hepatoma was induced in male Wistar rats (100–120 g) by feeding them with MeDAB as described previously[10]; the liver was quickly excised and tumors formed were dissected from the surrounding and necrotic liver tissues. Transplantable AH-109A hepatoma was inoculated subcutaneously into male Donryu rats (150–200 g) and harvested after 12 days. When liver development was being studied, Wistar fetuses and neonates were used. Regenerating liver was obtained from male Wistar rats (150–200 g) 24, 48 and 88 hr following partial hepatectomy. The procedure has already been described in detail.[11]

Preparation of Particulate Fraction All preparative operations described below were conducted at 4°. Liver and two hepatomas, each from 3–6 animals, were separately homogenized in 4 vol of $0.25M$ sucrose/$1mM$ EDTA in a glass/Teflon

homogenizer with 6 strokes. After centrifugation at 1,000*g* for 10 min, each supernatant was centrifuged at 105,000*g* for 1 hr, and the resulting pellet (the particulate fraction) was suspended in 1 ml/g tissue of 0.25*M* sucrose/1m*M* EDTA and used as the source of membrane sialidase.

Solubilization and Partial Purification of Membrane Sialidase Membrane sialidase was solubilized by adding deoxycholate and Triton X-100 to the above particulate suspension to final concentrations of 0.5 and 0.2% (w/v), respectively. After homogenization in a glass/Teflon homogenizer with 4 strokes, the mixture was centrifuged at 105,000*g* for 1 hr, and the resulting supernatant was saved. To further purify the enzyme, the supernatant fraction was brought to 50% saturation in ammonium sulfate and centrifuged. The pellet was then dissolved in a minimum volume of buffer A (20m*M* potassium phosphate, pH 6.8/1m*M* EDTA /0.1% Triton X-100) and passed through a Sephadex G-25 column. The desalted enzyme solution (100–150 mg in protein) was applied to a DEAE-cellulose column (1.5×12 cm) previously equilibrated with buffer A. The column was washed with buffer A and eluted with a linear 0–0.3*M* NaCl gradient in 150 ml of buffer A, collecting 5 ml fractions.

Sialidase Assay The standard assay mixture contained 100–200 nmol of mixed gangliosides as *Arthrobacter ureafaciens* sialidase-releasable sialic acid, 50m*M* sodium acetate (pH 4.5), 0.05% (w/v) sodium deoxycholate and 250 μl of enzyme in a final volume of 0.2 ml. After incubation at 37° for 1–3 hr, the reaction was stopped by immediate freezing. The mixture was then passed through an AG 1-X2 column as described previously,[7] and the amount of sialic acid released was determined by Warren's thiobarbituric acid method with corrections for interfering materials being made by measuring the absorption at 532 and 549 nm.[7, 12] Blanks for enzyme (with no exogenous substrate), substrate (with heat-inactivated enzyme) and assay conditions (with no 37° incubation) were simultaneously conducted and the values obtained were subtracted from the experimental values. Blanks for enzyme became unnecessary after the solubilization of the enzyme. Similarly, the AG 1-X2 treatment was no longer required after the DEAE-cellulose step. One unit of sialidase was defined as the amount of enzyme which catalyzed the release of 1 nmol of sialic acid/hr. Protein was determined by the method of Bradford.[13]

Results

The previous paper from this laboratory[6] demonstrated that in rat liver, the majority of the sialidase activity toward gangliosides is associated with the plasma membrane. Using isolated plasma membrane, the activity was highest at pH 4.5, and other substrates such as sialyllactose and orosomucoid were hydrolyzed less effectively than gangliosides.[6] To more fully characterize this membrane-associated and ganglioside-directing sialidase (membrane sialidase), the present studies employed the particulate fraction of tissue homogenate rather than isolated plasma membrane. By using the particulate fraction, a large loss of membrane sialidase activity during the isolation of plasma membrane can be avoided. Although the particulate fraction may be contaminated with a small amount of cytosolic sialidase, which is also capable of hydrolyzing gangliosides,[5] this contamination does not interfere with the assay of membrane sialidase, since cytosolic sialidase is almost inactive at

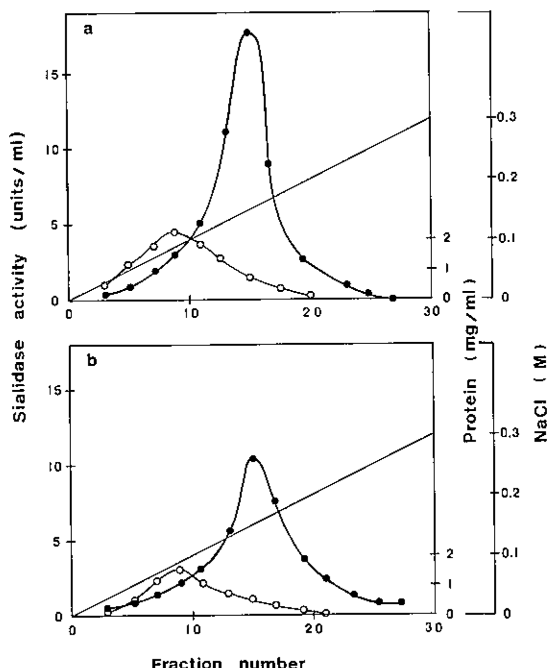

Fig. 1. Chromatography of membrane sialidases from the particulate fractions of liver (a) and MeDAB-induced hepatoma (b) on DEAE-cellulose. Solubilized particulate fraction obtained from 10 g of tissue was chromatographed on DEAE-cellulose, and the eluate, collected in fractions of 5 ml, was assayed for sialidase (●) and protein (○). For detailed procedures, see the text.

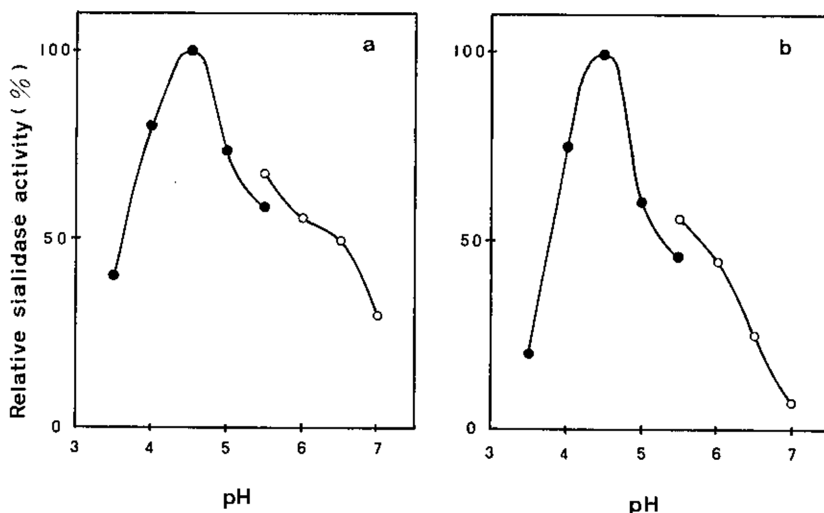

Fig. 2. Effect of pH on the activity of membrane sialidases from the particulate fractions of liver (a) and MeDAB-induced hepatoma (b). The enzyme was prepared by solubilization followed by DEAE-cellulose chromatography. Buffers used: pH 3.5–5.5, sodium acetate (●); pH 5.5–7.0, sodium cacodylate (○).

pH 4.5.[5] It should also be noted that after the solubilization step described below, DEAE-cellulose chromatography can totally remove cytosolic sialidase from membrane sialidase.

In attempts to solubilize membrane sialidase from the particulate fraction of rat liver, we found that 0.5% sodium deoxycholate plus 0.2% Triton X-100 solubilized the enzyme in an almost quantitative manner (91%). Under comparable conditions, the extents of solubilization by 0.5% sodium deoxycholate, 1% Triton X-100 and 3% octylglucoside were 22, 67 and 14%, respectively. Essentially the same results were obtained with the particulate fraction of rat hepatomas. The solubilized liver (Wistar) and hepatoma (MeDAB-induced) sialidases were then purified by chromatography on DEAE-cellulose. As shown in Fig. 1, the two enzymes were eluted from the column as a single peak at the same NaCl concentration. Since cytosolic sialidase was found to pass through this column (data not shown), the following qualitative studies were made with these partially purified preparations.

Figure 2 shows that the liver and hepatoma sialidases exhibit almost identical pH-activity curves, showing a maximum at pH 4.5 and a small shoulder at around pH 6.0. Both the liver and hepatoma enzymes required no metal for activity and hydrolyzed mixed gangliosides, 4-methylumbelliferyl-α-D-N-acetylneuraminic acid, 3′-sialyllactose and fetuin at relative rates of 5:2:2:1. The K_m for ganglioside GM_3 determined from double reciprocal plots of kinetic data was 2.17×10^{-5} and $2.08\times10^{-5}M$ for the liver and hepatoma enzymes, respectively. Taken together, these results suggest that hepatocarcinogenesis does not affect the qualitative aspect of membrane sialidase.

Then, to determine if any quantitative change in membrane sialidase occurred upon hepatocarcinogenesis, we assayed the particulate fractions of various types of liver and hepatoma at pH 4.5 using mixed gangliosides as substrate. The results obtained are summarized in Table I. Since relative tissue activities are little affected by solubilization, the tissue difference reported here most probably represents the tissue difference in enzyme amount. In Table I, sialidase activities are all expressed in terms of specific activity: this explains why the solubilized fraction exhibits higher sialidase activity than the particulate fraction.

Table I. Membrane-associated Sialidase Activity of Particulate Fraction from Rat Liver and Hepatomas

Tissues	Sialidase activity (units/mg)[a]	
	before solubilization	after solubilization
Wistar rats		
normal adult liver	11.6±0.2	13.7±0.3
MeDAB-induced hepatoma	5.8±0.1	9.7±0.1
fetal liver (−2 days)[b]	4.9±0.6	7.2±0.8
neonatal liver (+7 days)[b]	6.5±0.9	ND[d]
regenerating liver		
24 hr[c]	3.2±0.1	6.0±1.1
48 hr[c]	4.2±0.8	7.9±1.2
88 hr[c]	10.3±0.3	ND[d]
Donryu rats		
normal adult liver	12.7±0.2	ND[d]
AH-109A hepatoma	7.9±1.2	ND[d]

a) The assays were made under the standard conditions. The values given are means±SE of 5 experiments; 3 rats were killed for each experiment.
b) Before (−) or after (+) birth.
c) Hours after operation.
d) Not determined.

Table I demonstrates that the level of membrane sialidase is lowered in hepatomas as compared to control liver. This lowering is probably associated with cell proliferation, since partial hepatectomy also lowered membrane sialidase activity in a reversible manner. Table I also includes the data for fetal and neonatal livers: in the late-fetal stage, the level of membrane sialidase was 60% lower than the adult level. It is therefore suggested that the lowering of membrane sialidase activity in hepatomas is also among those alterations that can be classified as "oncofetal."

Discussion

The observation that rat liver possesses at least three types of sialidase differing in subcellular distribution and substrate preference[4–6] prompted us to investigate their activities and properties in experimental hepatomas. Such an investigation should facilitate the understanding not only of the mechanism for hypersialylation found in cancer cells[1–3] but also of the physiological role played by each sialidase. Our previous studies along this line,[7] however, dealt only with intralysosomal and cytosolic sialidases since the sialidase associated with the plasma membrane (membrane sialidase) was little characterized at that time. In the present work, membrane sialidase was solubilized and partially characterized. The studies that followed have made it clear that membrane sialidase from hepatomas is qualitatively identical to its non-cancerous counterpart but significantly lower in activity.

Although several workers have previously studied the neoplastic alterations of mammalian sialidase,[14–17] they apparently failed to reach a satisfactory conclusion, probably because they were unaware of the multiple nature of mammalian sialidase. Now, however, it is perfectly clear that rat liver contains three types of sialidase, of which one (intralysosomal sialidase) is increased but two others (cytosolic and membrane sialidases) are decreased upon hepatocarcinogenesis. We may be able to relate these changes to the physiological function of each sialidase. According to Merrit *et al.*,[18] N-2-fluorenylacetamide-induced hepatoma has a higher content of ganglioside-sialic acid than control liver. This is consistent with the present finding if membrane sialidase is assumed to regulate the level of cell-surface gangliosides. Interestingly, membrane sialidase exhibits much

greater affinity than cytosolic sialidase for the sialyl linkages of gangliosides (see above, and Ref. 5), although the optimal pH (4.5) seems to be unphysiological.

The present finding, on the other hand, apparently contrasts with that of Schengrund *et al.*,[19] who reported that exogenous gangliosides were desialylated by virally transformed fibroblasts and not by their non-transformed counterpart. The possibility exists, however, that this alteration resulted simply from an alteration in membrane structure, since they did not use detergents. Our finding, that is, the lower activity of membrane sialidase in hepatomas, has been shown to be retained even after solubilization of the enzyme by detergents.

ACKNOWLEDGMENTS

We thank Mr. K. Konno and Mrs. C. Ito for their skillful assistance. This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture.

(Received Aug. 19, 1987/Accepted Nov. 2, 1987)

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